Behavioural effects on marine amphipods exposed to silver ions and silver nanoparticles

Monizze Vannuci-Silva[1,2*], Shanelle Kohler[3], Gisela de A. Umbuzeiro[1,2], Alex T. Ford[3]

[1] Institute of Biology, University of Campinas, Campinas, SP, Brazil.

[2] School of Technology, University of Campinas, Limeira, SP, Brazil.

[3] Institute of Marine Sciences, University of Portsmouth, United Kingdom.

*Corresponding author:

E-mail address: monizze@hotmail.com

Tel: +5519981613188

Abstract

Behavioural responses to contaminants are an important endpoint in ecotoxicology because they link effects at biochemical or cellular levels to impacts on individual fitness. Due to the increasing use of silver in nanomaterials, studies of its effects on the behaviour of aquatic organisms are important to assess the risks of silver nanoparticles (AgNP) released into the environment. The aim of this work was to evaluate the behavioural effects of silver on the marine amphipod *Echinogammarus marinus* after exposure to $AgNO_3$ via water and AgCl or AgNP via food. Swimming activity of the amphipods was tracked during 6 min alternating dark and light conditions. Animals swam slower and responded less to light at higher concentrations of silver in the water. No differences were found in the behaviour of animals exposed via feeding up to 28 days, hence, longer exposure times may be required for the observation of effects. This is the first work to appraise behaviour effects of silver ions and AgNP on marine amphipods. Although the protocol has been successfully developed for this purpose, specimens appeared to habituate to test conditions during the experiments. Therefore, the need for further understanding of baseline behaviours in these model organisms is discussed.

Key-words: silver nanoparticles; response to light; swimming velocity; *Echinogammarus marinus*.

Capsule: Exposure to silver can lead to adverse effects on swimming behaviour in marine amphipods.

1. Introduction

Behaviour can be defined as the outcome of a sequence of neurophysiological events, which include the stimulation of sensory and motor neurons, muscular contractions and release of chemical messages (Lagadic et al., 1994). Additionally, behavioural responses integrate many cellular processes vital to an organism's survival and reproduction, reflecting biochemical and ecological consequences of toxic impact (Gerhardt, 1995). Several studies have used behavioural responses as tools for ecotoxicity testing and water quality monitoring (e.g. locomotor activity, response to light, ventilatory activity, feeding rate) because they are sensitive, fast, simple, and cost-effective to perform (Bakker et al., 1997; Wallace and Estephan, 2004). The organism's mobility and response to light are also behavioural markers with ecological relevance, as locomotion is essential to find food, escape predators and obtain mating success; whereas the response to a light stimulus is associated with predator avoidance (Bakker et al., 1997). Any pollutant that interferes with the mobility of an organism can, therefore, result in reduced fitness and 'ecological death' (Arce Funck et al., 2013; Scott and Sloman, 2004; Vellinger et al., 2012).

Silver (Ag) has been widely applied in nanomaterials (Fabrega et al., 2011; Musee, 2011; Purcell and Peters, 1998), mainly because of its broad-spectrum antimicrobial properties. As a consequence of its use, Ag can be released into the environment in a variety of compounds and forms (Morgan et al., 1997; Purcell and Peters, 1998), making it an element of environmental concern. Silver released into surface waters can reach toxic concentrations to the aquatic life - from picograms per litre to micrograms per litre (Purcell and Peters, 1998; Wood et al., 1999). Free silver ion (Ag^+) is the main species responsible for Ag toxicity in the aqueous phase, belonging to the

highest toxicity class together with Cd, Cr(VI) and Hg (Ratte, 1999 and references therein). However, for AgNP, it is still unclear if toxicity is due only to the dissolution of Ag^+ or whether the nanoparticles size, shape and defects in surface crystals contribute to their high toxicity (George et al., 2012; Vannuci-Silva et al., 2019). Once in the marine ecosystem, silver and its nanoparticles tend to agglomerate and precipitate to the sediment bottom (Forstner, 1983), leading to elevated exposure of benthic organisms such as amphipods.

Amphipods have been used in ecotoxicology studies for decades, being considered an excellent model for monitoring the health of aquatic biotopes and the effects of anthropogenic contaminants (Arce Funck et al., 2013; Felten et al., 2008; Gerhardt, 1995; Vellinger et al., 2012). They are ubiquitous to almost all water systems, abundant, and ecologically relevant. These marine crustaceans play a fundamental role in the ecosystem dynamics (Melo and Nipper, 2007) and have a short life and reproductive cycle. Furthermore, amphipods require little space and resources in the laboratory for husbandry and testing (Artal et al., 2017).

Several studies on the behavioural effects of metal exposure on invertebrates can be found in the literature, including freshwater amphipods, particularly *Gammarus* species. Altered behaviour responses after metal exposure on *Gammarus pulex* were reported by Gerhardt (1995), Felten et al. (2008) and Vellinger et al. (2012). Arce Funck et al. (2013) studied behavioural responses of the male freshwater amphipod *Gammarus fossarum* exposed to Ag (at 0, 0.5, 1, 2, and 4 µg L^{-1}) and found that locomotor and ventilatory activities were significantly reduced after 96h. However, studies on the behaviour of marine amphipods are still scarce. To the best of our knowledge, there is no information on Ag and AgNP effects on swimming behaviour in these organisms.

Echinogammarus marinus is a marine amphipod which inhabits a wide range of coastline in northwestern Europe, stretching from Norway to southern Portugal. Bossus et al. (2014) and Guler & Ford (2010) studied the behavioural effects of anti-depressants on this species and found significant alterations in swimming velocity, as well as photo and geotaxis responses, which could impact population-levels.

In this work, we investigated the behaviour effects on *E. marinus* after exposure to $AgNO_3$ via water and to AgNP or AgCl amended food. Both routes were evaluated because silver ions (Ag^+) are promptly absorbed via water, which implies full-time interaction between contaminants and organisms - whereas food exposure is intermittent – the reason why silver salts in solution are positive controls of silver exposure (Andreï et al., 2016; Pokhrel and Dubey, 2012). Complementarily, dietary assessment of Ag and AgNP exposure is especially relevant, given that contaminated food intake is one of the primary routes for silver and metallic nanomaterials incorporation (Petersen and Henry, 2012). The aims of this study were to understand behavioural alterations and develop an assay to evaluate sub-lethal effects of silver in the marine environment. We hypothesized that silver absorbed via contaminated water and food would lead to disruption on locomotion and light response in *E. marinus*.

2. Material and methods

2.1. Reagents, material and equipment

The reagents used in this work were: reverse osmosis water, concentrated nitric acid (HNO_3 - Fischer Scientific), sea salt (Red Sea®) and silver nitrate ($AgNO_3$ -

Fischer Scientific). Experimental equipment included plastic pots, plastic Petri dishes, plastic pipettes, tweezers, glassware and other materials generally used in biology laboratories. All glassware was decontaminated with 10% HNO_3 overnight prior to use. Other equipment used in this work were: salinity meter, pH meter, analytical scale, incubator and a DanioVision™ observation chamber connected to EthoVision®XT11.5 video tracking software (Track Sys, Nottingham, UK). The observation chamber supports an infrared (IR) camera located above the internal holder - which is backlit by both infrared and an additional white light - for an arena plate or small container (Kohler et al., 2018a). The IR camera (GigE Vision) is built-in DanioVision and is a high resolution (max 1280 * 960 px) camera with up to 60 frames per second (fps) rate.

2.2. *Echinogammarus marinus* sampling

The marine amphipods (*E. marinus*) were collected beneath seaweed and stones on the intertidal zone during low tide, at Langstone Harbour, Portsmouth, UK (50º47'23.13N 1º02'37.25W). The sampling site is characterised by areas of silt or gravel with larger rocks and debris, and the upper-intertidal zone is colonised predominantly by large aggregations of species of brown algae (Kohler et al., 2018b). According to the UK Government Environment Agency, in the 2000s, silver concentration in the Langstone waters was always lower than 1 µg L^{-1} (contains public sector information licensed under the Open Government Licence v3.0.; http://www.nationalarchives.gov.uk/doc/open-government-licence/version/3/). Organisms were manually caught and transported to the laboratory in a bucket with the algae *Ascophyllum nodosum* or *Fucus vesiculosis* filled with seawater from the sample site.

In the laboratory, organisms were counted and separated by size and gender. Males were visually selected under a stereo microscope and individualised from females by their proportionally larger gnathopods and differences in the hair structure on the telson. Adult males (size range between 1.5-2cm) were selected and acclimated in seawater from the sample site in incubators at 10°C for a minimum of 7 days. Constant aeration was provided via an air pump and air stone, and water renewal was performed in alternated days. Organisms were fed brown algae (*Ascophyllum nodosum*) collected from the sample site.

The organisms were kept under 24-hour dark photoperiod during the culture and the experiments. We focussed on reducing the circatidal rhythms which could interfere with the behavioural analysis, considering these rhythms cease after approximately 1 week (unpublished data). To standardise lighting regimes, we decided on a complete darkness acclimation period, as other studies used different light:dark periods, lux and light spectra (Kohler et al 2018a,b), which can also affect circadian rhythms.

2.3. Exposure experiments

2.3.1. Exposure via water

Experiment A1

Animals were exposed to silver nominal concentrations of 0, 5, 25 and 100 µg L^{-1} (from $AgNO_3$) for 96 hours, and their behaviour was analysed at the end of the exposure. Silver solutions were all freshly made for each experiment by dissolving $AgNO_3$ in artificial saline water, according to the targeted concentration of Ag. Artificial

saline water (salinity 33‰ ± 1) was prepared dissolving sea salt in reverse osmosis water. The organisms were kept individually in plastic pots with 100mL of saline water at 10°C for 24 hours in darkness, without aeration and feeding. Twenty replicates were used for each treatment. During the behaviour analysis, the animals were transferred to plastic Petri dishes filled with the same solution used in the exposure.

Experiment A2

The amphipods were exposed to 0, 5, 25, 100 and 200 µg of Ag (from $AgNO_3$) L^{-1}. Twenty organisms per treatment were kept in the same conditions as the experiment A1. However, the behaviour was also evaluated at 24, 48 and 72 hours, after which animals were returned to the exposure conditions (transferred back to the plastic pots in the incubator). After 96 hours of exposure, the behaviour was analysed in all animals for the last time.

2.3.2. Exposure via food

The control group was fed with basal diet formulated to provide ca. 40% protein and 6% lipid. Silver nanoparticles (Silver nano <100 nm, Sigma-Aldrich) were incorporated into the basal ingredients of the diet by adding the unmodified powdered form of NP to the feed pellets as described by Merrifield (2013). The AgNP were identical and from the same batch as reported in Bradford et al. (2009) and Merrifield et al. (2013), with a mean particle diameter of 58.6 ±18.6 nm (mean ± S.D., n = 64). As a positive control, and to distinguish between nanoparticulate and elemental forms, Ag salt (as AgCl, Sigma-Aldrich) was added to the basal ingredients to produce diets for treatments labelled as AgCl. The Ag concentrations in prepared diets were determined by Graphite

187 Furnace Atomic Absorption Spectroscopy (GFAAS), reaching 195 mg kg^{-1} and 155 mg
188 kg^{-1} for AgCl and AgNP, respectively.
189
190 *Experiment B1*
191 Animals were individually allocated in plastic containers with 100 mL of
192 artificial saline water (salinity 33‰ ± 1) and were fed with control, AgNP or AgCl pellets
193 in alternate days. The animals were fed *ad libitum*, however, we determined the amount
194 of total Ag for each diet and we observed that animals fed with control, AgCl and AgNP
195 pellets ingested approximately the same amount of food. Fifteen replicates were used for
196 each treatment. After 1 hour of feeding, organisms were transferred into a new container
197 with fresh seawater to ensure that the exposure was strictly via food. Exposure times were
198 7, 14 and 28 days and, after the end of each of them, behaviour analysis was performed.
199 Temperature and photoperiod were the same as the exposure via water (10°C and 24-hour
200 darkness) and without aeration.
201
202 *Experiment B2*
203 Exposure conditions were the same as experiment B1, except that 20
204 replicates were used and the behaviour of the same individual was analysed after 7, 14,
205 21 and 28 days of exposure. Following behaviour analysis, animals were returned to the
206 exposure conditions (transferred back to the plastic pots in the incubator).
207
208 **2.4. Behaviour analysis**
209
210 Behavioural assays were performed following exposure using a
211 DanionVision™ observation chamber with EthoVision® XT software, as described in

previous works (Kohler et al., 2018a, 2018b). Organisms were individually placed - using a plastic spoon to reduce handling stress - in a behavioural chamber containing artificial saline water to a depth of 15mm. The water depth allowed for free horizontal swimming but limited vertical swimming. Individuals were allocated a 1-minute acclimation time to assay conditions prior to recording. The velocity (cm/s) measurements of amphipods were recorded every 0.1 second(s) during a period of 3-minutes dark and 3-minutes light (2000 lux). The swimming velocity was chosen because previous works mention this parameter as the most affected by metal exposure (Arce Funck et al., 2013; Felten et al., 2008; Lebrun et al., 2017; Mills et al., 2006; Wallace and Estephan, 2004). Following the study by Kohler et al. (2018b) all data was processed into 10-seconds bins. Heat-maps were made, enabling a visual representation of periods when the amphipods were very active or inactive during dark (lights off) and light (lights on) phases.

2.5. Statistical analyses

Statistical analyses were performed using IBM SPSS® Statistics 24. Linear Mixed-Effects (LME) statistical analysis was performed for behaviour data using velocity as dependent variable and time (bins), concentrations and exposure time as factors. Extreme anomalous values generated by the loss of tracking by the software were excluded from the data analysis (as defined by values > median±3*IQR), which never accounted for more than 3% of data points. Tukey's pairwise comparisons were used for Post Hoc analysis. P values of <0.05 were considered significant.

3. Results

237

238 **3.1. Behaviour analysis**

239

240 **3.1.1. Exposure via water**

241

242 *Experiment A1*

243 The mean swimming velocity peaks just after lights on and it gradually comes
244 back to lower values. The velocity peak was inversely proportional to Ag concentration
245 in water (Figure 1). The faster velocity peaks were noticeable for the control organisms
246 and the lowest concentration (5 µg L^{-1}), which showed no significant differences between
247 their responses to light (p=0.973). However, velocity after lights on in 100 and 25 ug L^{-1}
248 treatments were significantly different than control (p=0,007 and <0.001, respectively)
249 and 5 ug L^{-1} (p=0.028 and <0.001, respectively). The measurements between treatments
250 were not different (F(3, 72.09)=1.50, p=0.221), but the measurements between dark/light
251 phase (F(35, 2484.2)=1.50, p<0.001) and the interaction between treatments and
252 dark/light phase were different (F(105, 2484.2)=3.82, p<0.001).

253

254 *Experiment A2*

255 Experiment A2 applied the same concentrations as experiment A1 and an
256 additional 200µg L^{-1} concentration. The behaviour analysis was performed after 24, 48,
257 72 and 96 hours of exposure, using the same specimens. No significant increase in the
258 swimming velocity just after lights on was observed in all treatments, except the 24-h and
259 96-h exposures to 5 µg L^{-1}, in which a peak in velocity was detected (Figure 2). There
260 was difference in the interaction between time (bins) and concentration, but no difference

was found in the interaction between time (bins), concentration and exposure time (hours) (Table 1).

Figure 3 shows the heat map of swimming velocity during dark and light phases of organisms exposed to Ag via water for experiments A1 and A2. The heat map for each treatment represents an average of the replicates that were used. For both experiments, animals exposed to the highest concentrations tended to swim faster during the dark phase (dark yellow squares in heat map). After lights on, high-velocity peaks were detected in experiment A1 for the control and 5 µg L^{-1} concentration in water (dark red squares in heat map), which were not so evident in experiment A2.

3.1.2. Exposure via food

Experiment B1

No difference between treatments (control, AgCl and AgNP food) was found. On the other hand, there was difference in exposure time factor (Table 2) that was induced by the decrease in the response to light after 14 and 28 days of exposure compared to 7 days ($F(2, 73.43)=20.55$, $p<0.001$) (Figure 4). Unlike the results of the A1 experiment, a negative effect in the response to light was not observed in animals fed with contaminated food (Table 2).

Experiment B2

Statistical analyses for experiment B2 are described in Table 3. As well as experiment B1, there was no difference between treatments in the last experiment, but there was difference in time (bins) and exposure time (days) individual factors. There was

a significant difference in the interactions between time (bins) and treatment, and time (bins) and exposure time (days). The difference found in the exposure time was clearly leading by the longest exposure (28 days) when the swimming velocity decrease compared to other treatments, either for dark or light (Figure 5).

Figure 6 shows the heat map of swimming velocity during dark and light phases of organisms exposed to Ag via food in the experiment B1 and B2. The heat map for each treatment represents an average of the replicates that were used. It is noticeable a decrease in the mean swimming velocity after lights on related to longer exposure times.

4. Discussion

Movement is a highly ecologically relevant behavioural marker since locomotion is required to find food, escape predation and obtain mates (Arce Funck et al., 2013). Therefore, if metals interfere with locomotor activity, they will likely reduce fitness and could lead to "ecological death" of an organism (Scott and Sloman, 2004). There are some studies reporting the possible physiological and biochemical mechanisms behind the metal effects on behaviour (Felten et al., 2008; J. Lawrence and Poulter, 1998; Khoury et al., 2009). The difference in the mean velocity between treatments in the first seconds of the light phase found in the experiment A1 corroborates the literature that reports aquatic amphipods as negatively phototactic organisms (Brundin, 1913).

The organisms analysed multiple times (experiments A2 and B2) did not show such a pronounced response as those which were tracked a single time at the end of the exposure. This suggests that the animals were becoming habituated to the daily assays.

Habituation is a common response to repeated assays and can be defined as a decline in response to novelty or stress (Biro, 2012). Our results corroborate previous works in which this phenomenon has been described during hormonal, physiological and behavioural experiments (Biro, 2012; Martin and Réale, 2008; Romero, 2004; Wong et al., 2010). Comparing experiments A1 and A2, the baseline swimming velocity of the animals in the latter was faster. In the first experiment, animals swam under 0.5cm/s in the dark, while in the second experiment baseline velocity reached 2cm/s after 96hrs. It is worth highlighting, however, that in the 2nd experiment individuals were being moved around daily, whereas in the 1st experiment remained static, which could explain baseline variation in the experiments. Additionally, over the 24-96h period, the general swimming rate decreased. Exposures via food support this hypothesis, once the organisms were manipulated in alternate days, with analogous effects over exposure time. Hence, it is possible to speculate that increasing disturbance adds variability to the data, potentially masking the effects of pollutants. To avoid habituation in future behaviours assays, we strongly recommended not to use the same individual for subsequent trackings. Additionally, previous studies could be performed to constrain the suitable time interval in between trackings to prevent habituation.

There is a lack of information on Ag effects on organisms' behaviour in literature, especially for marine amphipods. Earlier studies were performed using the freshwater amphipod *Gammarus* sp. and evaluated exposure to cadmium, copper, nickel, lead, zinc and silver via water (Arce Funck et al., 2013; Felten et al., 2008; Lebrun et al., 2017; Mills et al., 2006; Wallace and Estephan, 2004). In all studies, locomotion was significantly affected by each exposure, except for copper. However, the results reported in those studies and our findings are not comparable due to the difference in the bioavailability of Ag as a free ion in fresh and seawater. It is known that Ag is less toxic

to seawater than freshwater organisms, mainly because of its complexation with Cl^- in the saline environment, which makes Ag unavailable. Thus, the effects of silver contamination in marine environments are expected to occur in higher concentrations than in freshwater (Bury et al., 2002; Luoma et al., 1995).

Swimming behaviour was affected when amphipods were exposed to high concentrations of Ag via water after 96-hour exposure (experiment A1, Figure 1). The organisms showed similar and constant velocity during the dark phase for all treatments. However, during the light phase, it was observed a reduction of 1.7%, 26.0% and 54.8% in the swimming velocity of organisms exposed for 96 hours to 5, 25 and 100 µg L^{-1}, respectively, when compared to control during the 3 minutes of illumination. The decrease in the swimming velocity was greater for the first 20 seconds of lights on, when the negative effect was 33.7% and 58.9% in the velocity for the 25 and 100 µg L^{-1} treatments. Nevertheless, there was an increase of 12.9% for the treatment 5 µg L^{-1} when compared to control (Figure 1). Arce Funck et al. (2013) found that, after 48 hours of exposure, the locomotor activity decreased by 13%, 48% and 61% in amphipods exposed to 0.5, 1 and 2 µg Ag L^{-1}, respectively. A higher and significant decrease was noted by the same authors following a 96 hours period, when exposure to 0.5 and 1 µg Ag L^{-1} led to a reduction of 75% and 61% in the locomotor activity. Therefore, the decrease in locomotion observed in marine amphipods exposed to $AgNO_3$ is consistent with the results by Arce Funck et al. (2013) using freshwater species. Despite that, comparing the percentage of decrease, freshwater amphipods seem to be more sensitive to Ag exposure than marine species, probably due to the reduced bioavailability of Ag as a free ion (Ag^+) in seawater. No studies evaluating silver effects on swimming behaviour of marine organisms were found in the literature.

Alterations in swimming behaviour of *E. marinus* were not observed following exposure to AgCl and AgNP via food. No studies were found in the literature investigating exposure to silver and/or silver nanoparticles via food and its effects on behaviour in aquatic organisms. Few studies reported adverse effects on behaviour on freshwater crustaceans *Daphnia magna* and *Gammarus roeseli* and the *Danio rerio* larval fish after AgNP exposure via water (Andreï et al., 2016; Asghari et al., 2012; Pokhrel and Dubey, 2012; Powers et al., 2011). Our findings do not corroborate the effects of AgNP exposure reported by the studies above, however, it should be considered that the exposure route was different. Also, the period for dietary exposure should be considered. Ag uptake rates and concentration in the haemolymph of the marine amphipod *Parhyale hawaiensis* were described by Vannuci-Silva et al. (2018; 2019). Authors indicated that animals exposed via food containing AgCl would require more than 100 days to uptake similar amounts of silver absorbed by organisms exposed via water for only 96 hours (Vannuci-Silva et al., 2019). For food containing AgNP, a 30 to 40 days period would be necessary to reach the same silver internal concentration than a 96-hour exposure via water. Hence, over 28 days would be required for the observation of behavioural effects through food exposure. Nonetheless, long term studies should be mindful of animal habituation, which could potentially mask toxicological responses within the experimental design.

5. Conclusions

Silver affected swimming and response to light behaviour on *E. marinus* exposed to $AgNO_3$ via water. Animals swam slower and responded less to light proportionally to the increase of Ag in the water. There was no difference in response to

light between treatments for exposure to AgCl or AgNP incorporated in the food after 28 days. Perhaps, longer exposure times would be required for the observation of effects.

This was the first work to assess behaviour effects of silver and silver nanoparticles on a marine amphipod. Although the protocol has been successfully developed for its purpose, we observed that the specimens appeared to habituate to test conditions during the experiments. Therefore, caution should be taken when interpreting data from these novel behavioural assays, given the changes in the laboratory animals' behaviours over time, which may mask or enhance the effects of the toxicants.

Acknowledgements

The authors thank FAPESP and CAPES (grants 2013/26301-7 and 2016/19635-4, São Paulo Research Foundation - FAPESP) for the financial support and the UK Environment Agency for providing the water quality data of Langstone Harbour.

Funding

This work was supported by the São Paulo Research Foundation (FAPESP) and the Coordination for the Improvement of Higher Education Personnel (CAPES) [grant numbers 2013/26301-7 and 2016/19635-4].

Conflict of interest

The authors declare no conflict of interest.

410 **References**

411

412 Andreï, J., Pain-Devin, S., Felten, V., Devin, S., Giambérini, L., Mehennaoui, K.,
413 Cambier, S., Gutleb, A.C., Guérold, F., 2016. Silver nanoparticles impact the
414 functional role of *Gammarus roeseli* (Crustacea Amphipoda). Environ. Pollut. 208,
415 608–618. doi:10.1016/j.envpol.2015.10.036
416 Arce Funck, J., Danger, M., Gismondi, E., Cossu-Leguille, C., Guerold, F., Felten, V.,
417 2013. Behavioural and physiological responses of *Gammarus fossarum* (Crustacea
418 Amphipoda) exposed to silver. Aquat. Toxicol. 142–143, 73–84.
419 doi:10.1016/j.aquatox.2013.07.012
420 Artal, M.C., dos Santos, A., Henry, T.B., Umbuzeiro, G. de A., 2017. Development of
421 an acute toxicity test with the tropical marine amphipod *Parhyale hawaiensis.*
422 Ecotoxicology. doi:10.1007/s10646-017-1875-3
423 Asghari, S., Johari, S.A., Lee, J.H., Kim, Y.S., Jeon, Y.B., Choi, H.J., Moon, M.C., Yu,
424 I.J., 2012. Toxicity of various silver nanoparticles compared to silver ions in
425 *Daphnia magna*. J. Nanobiotechnology 10, 14. doi:10.1186/1477-3155-10-14
426 Bakker, T.C.M., Mazzi, D., Zala, S., 1997. Parasite-Induced Changes in Behavior and
427 Color Make G*ammarus pulex* More Prone to Fish Predation. Ecology 78, 1098–
428 1104.
429 Biro, P.A., 2012. Do rapid assays predict repeatability in labile (behavioural) traits?
430 Anim. Behav. 83, 1295–1300. doi:10.1016/j.anbehav.2012.01.036
431 Bossus, M.C., Guler, Y.Z., Short, S.J., Morrison, E.R., Ford, A.T., 2014. Behavioural
432 and transcriptional changes in the amphipod *Echinogammarus marinus* exposed to
433 two antidepressants, fluoxetine and sertraline. Aquat. Toxicol. 151, 46–56.
434 doi:10.1016/j.aquatox.2013.11.025

435 Bradford, A., Handy, R.D., Readman, J.W., Atfield, A., Muhling, M., 2009. Impact of
436 silver nanoparticle contamination on the genetic diversity of natural bacterial
437 assemblage in estuarine sediments. Environ. Sci. Technol. 43, 4530–4536.
438 Brundin, T.M., 1913. Light reactions of terrestrial amphipods. J. Anim. Behav. 3, 334–
439 352. doi:10.1037/h0072711
440 Bury, N.R., Shaw, J., Glover, C., Hogstrand, C., 2002. Derivation of a toxicity-based
441 model to predict how water chemistry influences silver toxicity to invertebrates.
442 Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 133, 259–270.
443 doi:10.1016/S1532-0456(02)00096-0
444 Fabrega, J., Luoma, S.N., Tyler, C.R., Galloway, T.S., Lead, J.R., 2011. Silver
445 nanoparticles: Behaviour and effects in the aquatic environment. Environ. Int. 37,
446 517–531. doi:10.1016/j.envint.2010.10.012
447 Felten, V., Charmantier, G., Mons, R., Geffard, a., Rousselle, P., Coquery, M., Garric,
448 J., Geffard, O., 2008. Physiological and behavioural responses of *Gammarus pulex*
449 (Crustacea: Amphipoda) exposed to cadmium. Aquat. Toxicol. 86, 413–425.
450 doi:10.1016/j.aquatox.2007.12.002
451 Forstner, U., 1983. Metal transfer between solid and aqueous phases. In: U. Forstner &
452 G.T.W. Wittmann, in: Metal Pollution in the Aquatic Environmental. Berlin
453 Heidelberg:Springer-Verlag, Berlin, p. 197.
454 George, S., Lin, Sijie, Ji, Z., Thomas, C.R., Li, L., Mecklenburg, M., Meng, H., Wang,
455 X., Zhang, H., Xia, T., Hohman, J.N., Lin, Shuo, Zink, J.I., Weiss, P.S., Nel, A.E.,
456 2012. Surface defects on plate-shaped silver nanoparticles contribute to its hazard
457 potential in a fish gill cell line and zebrafish embryos. ACS Nano 6, 3745–3759.
458 doi:10.1021/nn204671v
459 Gerhardt, A., 1995. Monitoring behavioural responses to metals in *Gammarus pulex*

(L.) (Crustacea) with impedance conversion. Environ. Sci. Pollut. Res. 2, 15–23. doi:10.1007/BF02987506

Guler, Y., Ford, A.T., 2010. Anti-depressants make amphipods see the light. Aquat. Toxicol. 99, 397–404. doi:10.1016/j.aquatox.2010.05.019

J. Lawrence, A., Poulter, C., 1998. Development of a sub-lethal pollution bioassay using the estuarine amphipod *Gammarus duebeni*. Water Res. 32, 569–578. doi:10.1016/S0043-1354(97)00306-0

Khoury, J.N., Powers, E., Patnaik, P., Wallace, W.G., 2009. Relating disparity in competitive foraging behavior between two populations of Fiddler crabs to the subcellular partitioning of metals. Arch. Environ. Contam. Toxicol. 56, 489–499. doi:10.1007/s00244-008-9270-y

Kohler, S.A., Parker, M.O., Ford, A.T., 2018a. Shape and size of the arenas affect amphipod behaviours : implications for ecotoxicology. Peer J 6, 1–20. doi:10.7717/peerj.5271

Kohler, S.A., Parker, M.O., Ford, A.T., 2018b. Species-specific behaviours in amphipods highlight the need for understanding baseline behaviours in ecotoxicology. Aquat. Toxicol. 202, 173–180. doi:10.1016/j.aquatox.2018.07.013

Lagadic, L., Caquet, T., Ramade, F., 1994. The role of biomarkers in environmental assessment (5). Invertebrate populations and communities. Ecotoxicology 3, 193–208. doi:10.1007/BF00117084

Lebrun, J.D., Uher, E., Fechner, L.C., 2017. Behavioural and biochemical responses to metals tested alone or in mixture (Cd-Cu-Ni-Pb-Zn) in *Gammarus fossarum*: From a multi-biomarker approach to modelling metal mixture toxicity. Aquat. Toxicol. 193, 160–167. doi:10.1016/j.aquatox.2017.10.018

Luoma, S.N., Ho, Y.B., Bryan, G.W., 1995. Fate, bioavailability and toxicity of silver in

485 estuarine environments. Mar. Pollut. Bull. 31, 44–54. doi:10.1016/0025-
486 326X(95)00081-W
487 Martin, J.G.A., Réale, D., 2008. Temperament, risk assessment and habituation to
488 novelty in eastern chipmunks, Tamias striatus. Anim. Behav. 75, 309–318.
489 doi:10.1016/j.anbehav.2007.05.026
490 Melo, S., Nipper, M., 2007. Sediment toxicity tests using the burrowing amphipod
491 *Tiburonella viscana* (Amphipoda: Platyischnopidae). Ecotoxicol. Environ. Saf. 66,
492 412–20.
493 Merrifield, D.L., Shaw, B.J., Harper, G.M., Saoud, I.P., Davies, S.J., Handy, R.D.,
494 Henry, T.B., 2013. Ingestion of metal-nanoparticle contaminated food disrupts
495 endogenous microbiota in zebrafish (*Danio rerio*). Environ. Pollut. 174, 157–163.
496 doi:10.1016/j.envpol.2012.11.017
497 Mills, C.L., Shukla, D.H., Compton, G.J., 2006. Development of a new low cost high
498 sensitivity system for behavioural ecotoxicity testing. Aquat. Toxicol. 77, 197–
499 201. doi:10.1016/j.aquatox.2005.12.003
500 Morgan, I.J., Henry, R.P., Wood, C.M., 1997. The mechanism of acute silver nitrate
501 toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill
502 Na+ and Cl- transport. Aquat. Toxicol. 38, 145–163. doi:10.1016/S0166-
503 445X(96)00835-1
504 Musee, N., 2011. Nanowastes and the environment: Potential new waste management
505 paradigm. Environ. Int. 37, 112–128. doi:10.1016/j.envint.2010.08.005
506 Petersen, E.J., Henry, T.B., 2012. Methodological considerations for testing the
507 ecotoxicity of carbon nanotubes and fullerenes: Review. Environ. Toxicol. Chem.
508 31, 60–72. doi:10.1002/etc.710
509 Pokhrel, L.R., Dubey, B., 2012. Potential impact of low-concentration silver

510 nanoparticles on predator-prey interactions between predatory dragonfly nymphs
511 and *Daphnia magna* as a prey. Environ. Sci. Technol. 46, 7755–7762.
512 doi:10.1021/es204055c
513 Powers, C.M., Slotkin, T.A., Seidler, F.J., Badireddy, A.R., Padilla, S., 2011. Silver
514 nanoparticles alter zebrafish development and larval behavior: Distinct roles for
515 particle size, coating and composition. Neurotoxicol. Teratol. 33, 708–714.
516 doi:10.1016/j.ntt.2011.02.002
517 Purcell, T.W., Peters, J.J., 1998. Sources of silver in the environment. Environ. Toxicol.
518 Chem. 17, 539–546. doi:10.1002/etc.5620170404
519 Ratte, H.T., 1999. Bioaccumulation and toxicity of silver compounds: A review.
520 Environ. Toxicol. Chem. 18, 89–108. doi:Doi 10.1897/1551-
521 5028(1999)018<0089:Batosc>2.3.Co;2
522 Romero, L.M., 2004. Physiological stress in ecology: Lessons from biomedical
523 research. Trends Ecol. Evol. 19, 249–255. doi:10.1016/j.tree.2004.03.008
524 Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex
525 fish behaviour: Integrating behavioural and physiological indicators of toxicity.
526 Aquat. Toxicol. 68, 369–392. doi:10.1016/j.aquatox.2004.03.016
527 Vannuci-Silva, M., Cadore, S., Henry, T.B., Umbuzeiro, G. de A., 2019. Higher Ag
528 bioavailability after nanoparticles dietary exposure in marine amphipods. Environ.
529 Toxicol. Chem. doi:10.1002/etc.4359
530 Vannuci-Silva, M., do Nascimento da Silva, E., Coletty, M., dos Santos, A., Silva, F.,
531 Umbuzeiro, G.D.A., Cadore, S., 2018. GFAAS and ICP-MS determination of Ag
532 and Cu in the haemolymph of a millimetric marine crustacean (*Parhyale*
533 *hawaiensis*) as a Tool in Ecotoxicology. At. Spectrosc. 39, 67–74.
534 Vellinger, C., Felten, V., Sornom, P., Rousselle, P., Beisel, J.N., Usseglio-Polatera, P.,

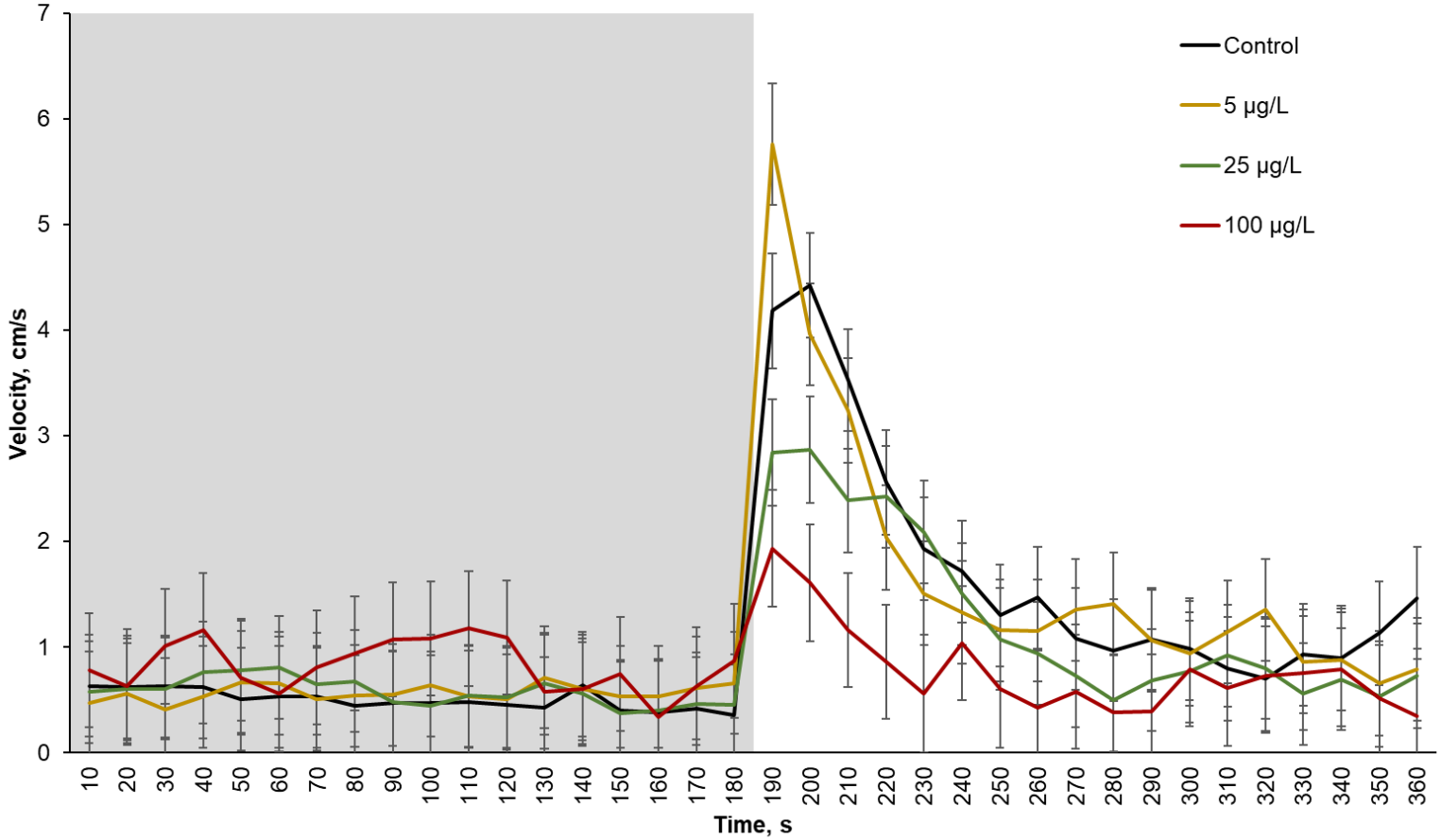
2012. Behavioural and physiological responses of *Gammarus pulex* exposed to cadmium and arsenate at three temperatures: Individual and combined effects. PLoS One 7. doi:10.1371/journal.pone.0039153

Wallace, W.G., Estephan, A., 2004. Differential susceptibility of horizontal and vertical swimming activity to cadmium exposure in a gammaridean amphipod (*Gammarus lawrencianus*). Aquat. Toxicol. 69, 289–297. doi:10.1016/j.aquatox.2004.05.010

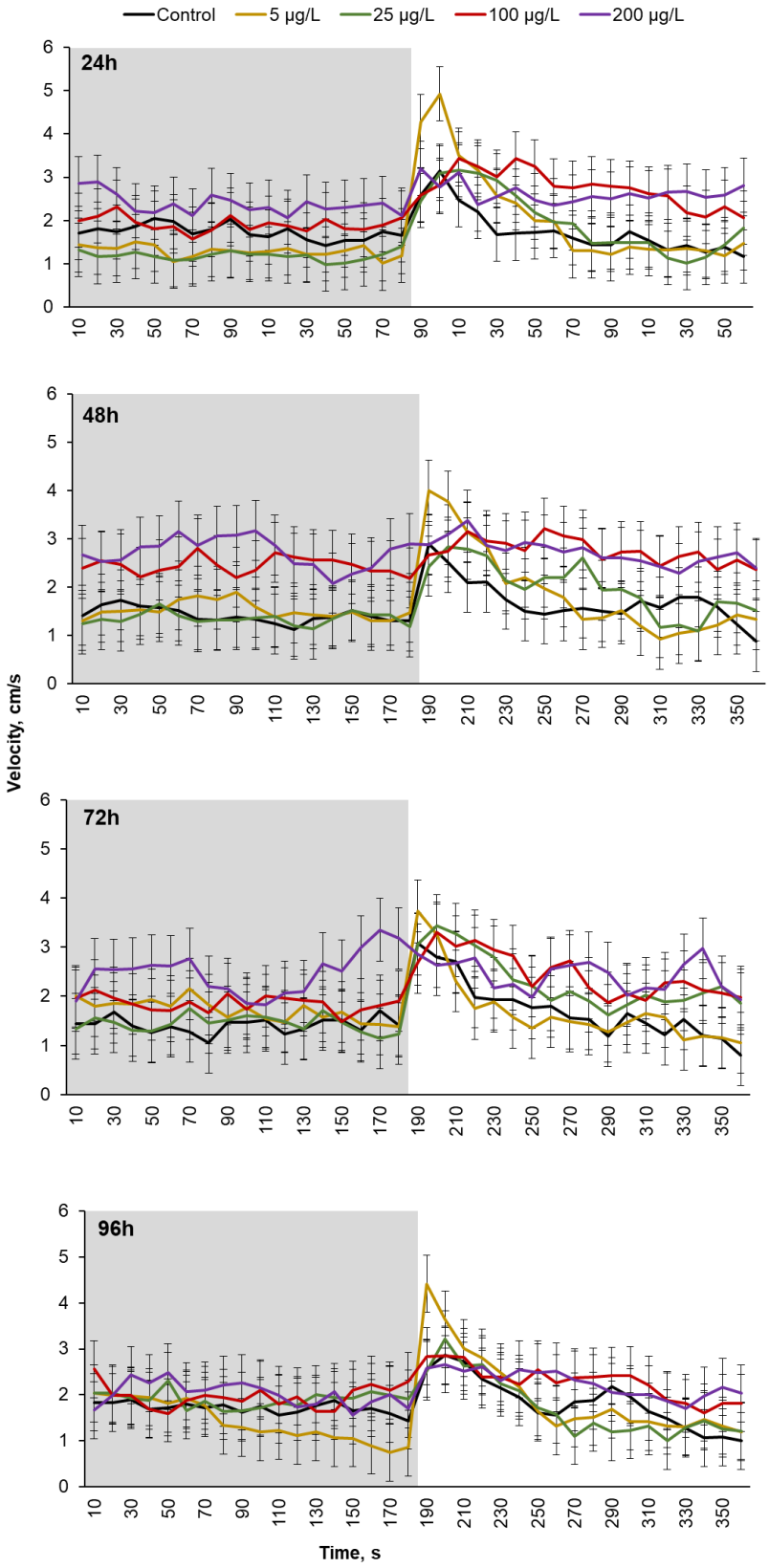
Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suciu, C., Tan, J., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K.M., Stewart, A., Gilder, T., Beeson, E., Zapolsky, I., Wu, N., Cachat, J., Kalueff, A. V., 2010. Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). Behav. Brain Res. 208, 450–457. doi:10.1016/j.bbr.2009.12.023

Wood, C.M., Playle, R.C., Hogstrand, C., 1999. Physiology and modeling of mechanisms of silver uptake and toxicity in fish. Environ. Toxicol. Chem. 18, 71–83. doi:10.1897/1551-5028(1999)018<0071:pamomo>2.3.co;2

551 **Figures**



552
553 **Figure 1.** *Echinogammarus marinus* 10 seconds bin mean velocity (n=20) after exposure via water for 96 hours. The grey background represents
554 dark phase and the white background represents light phase.



555

556 **Figure 2.** *Echinogammarus marinus* 10 seconds bin mean velocity (n=20) after exposure
557 via water for 24, 48, 72 and 96 hours. The grey background represents dark phase and the
558 white background represents light phase.

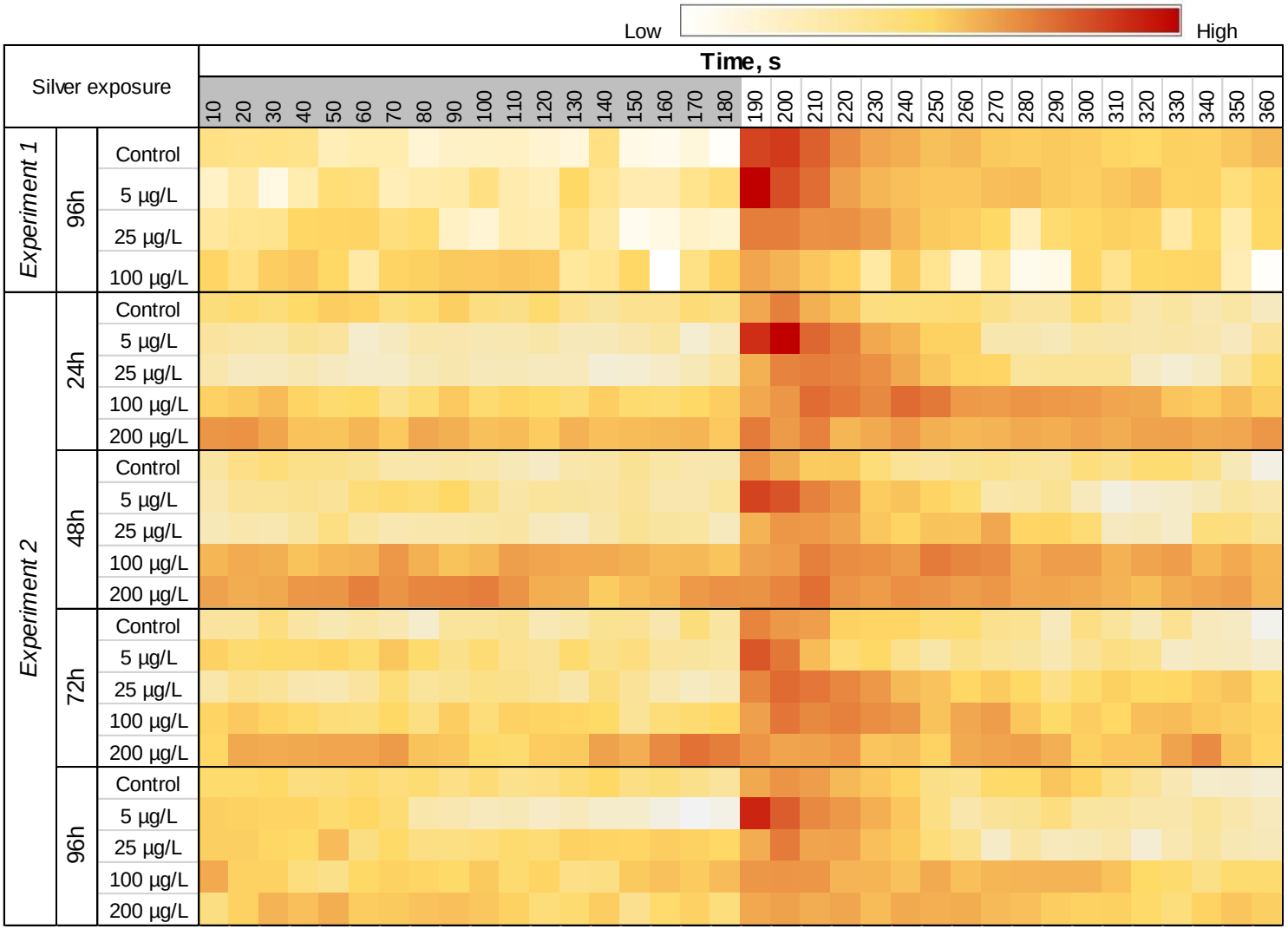
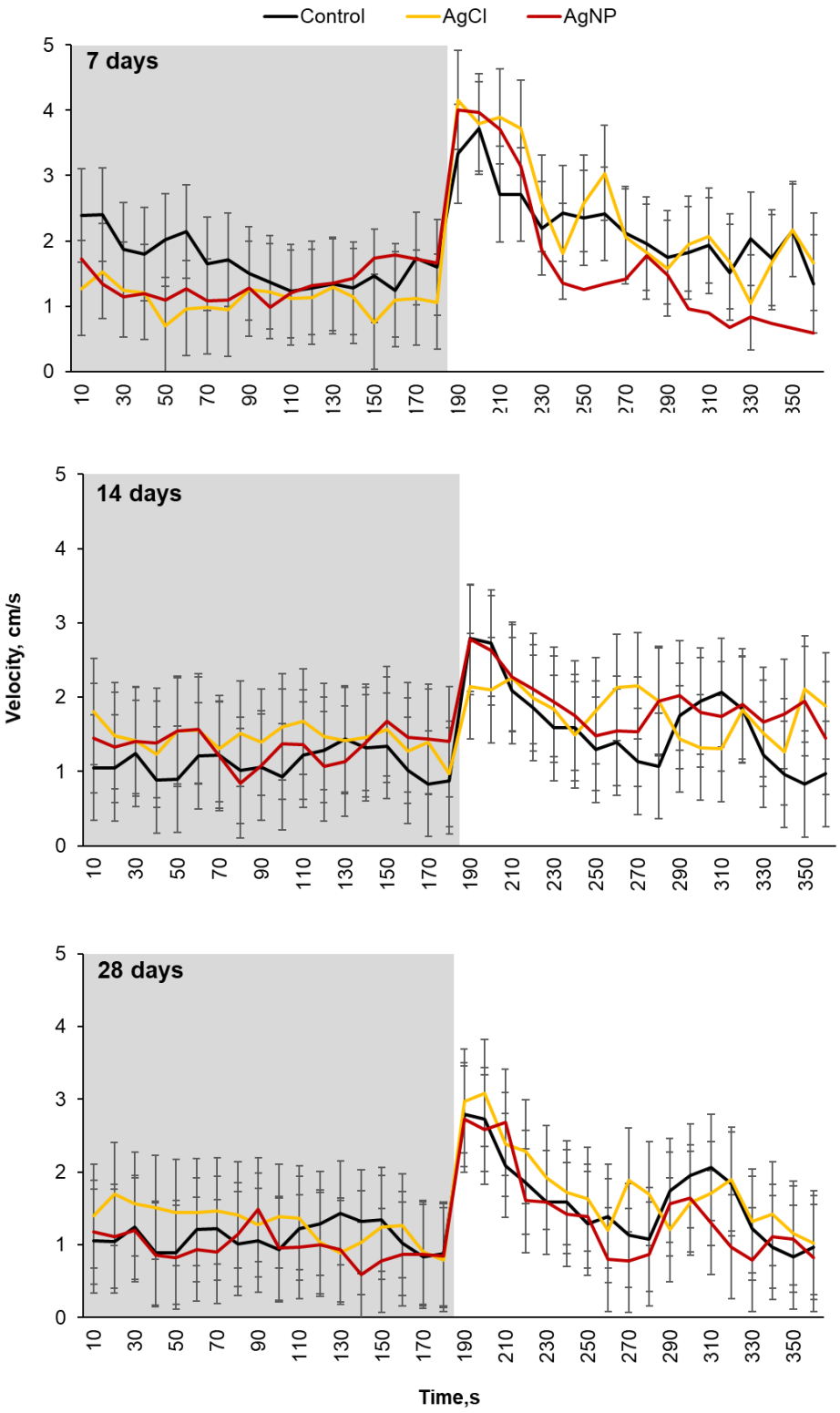


Figure 3. Average heat map of *Echinogammarus marinus* swimming velocity after exposure to Ag via water.



561

562 **Figure 4.** *Echinogammarus marinus* 10 seconds bin mean velocity (n=15) after exposure
563 via food for 7, 14 and 28 days. The grey background represents dark phase and the white
564 background represents light phase.
565

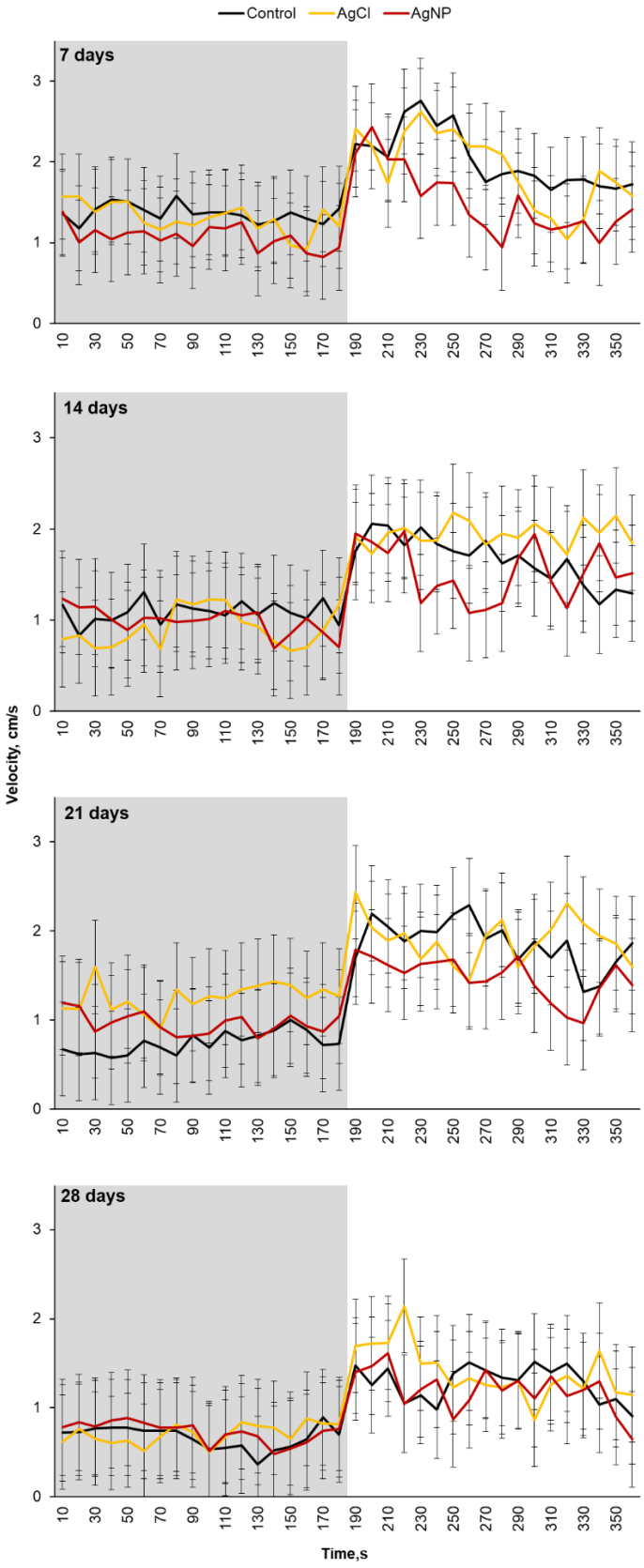
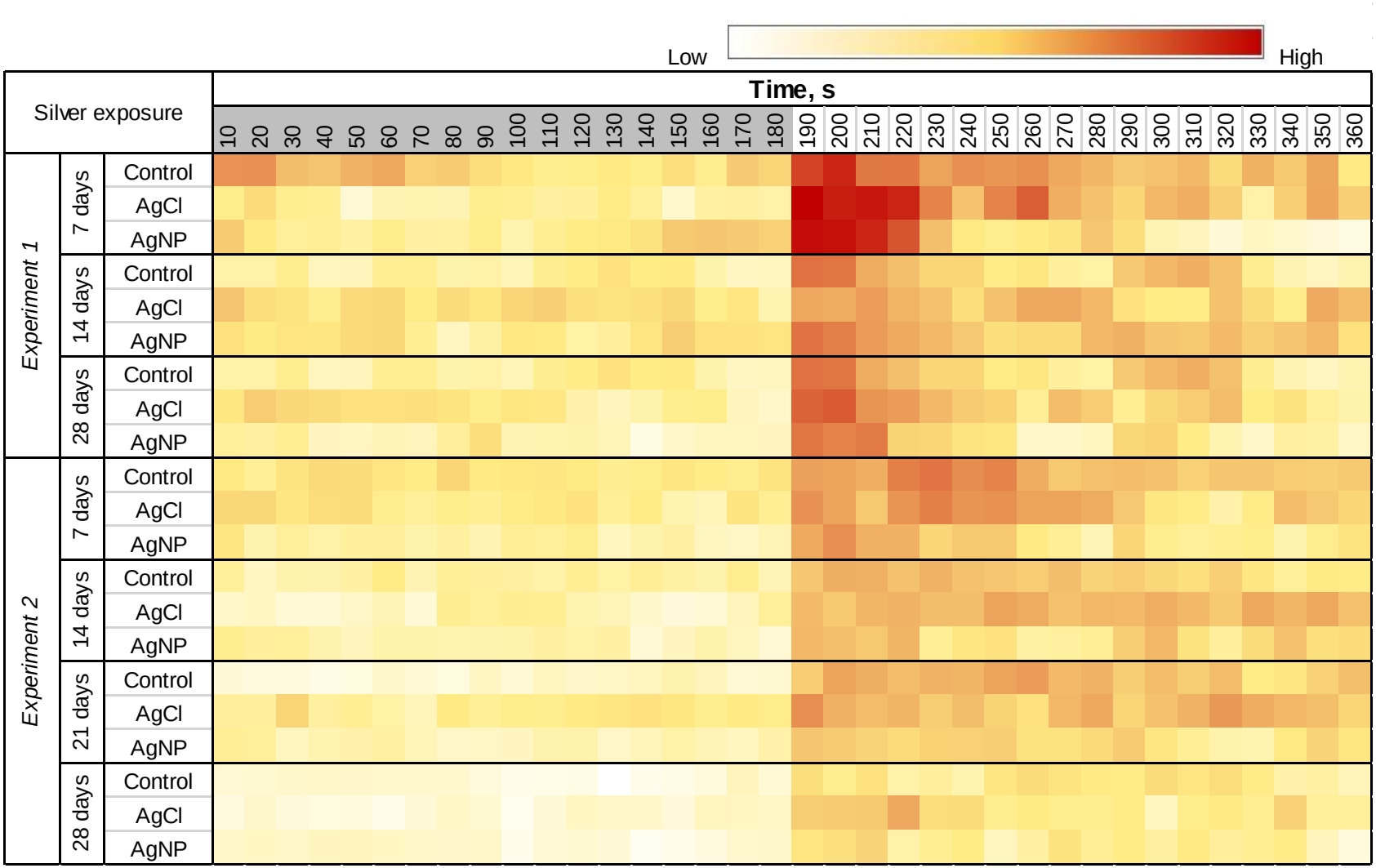


Figure 5. *Echinogammarus marinus* 10 seconds bin mean velocity (n=20) after exposure via food for 7, 14, 21 and 28 days. The grey background represents dark phase and the white background represents light phase.



570

571 **Figure 6.** Average heat map of *Echinogammarus marinus* swimming velocity after exposure to Ag via food (control, AgCl and AgNP).

572 **Tables**

573

574

Table 1. LME statistical analysis for experiment A2. Velocity (cm/s) as dependent variable.

Factors	Numerator df	Denominator df	F	Sig.
Bins, s	35	13281.2	38.1	< 0.001
Concentration, µg L^{-1}	4	379.9	21	< 0.001
Exposure time, h	3	379.9	0.6	0.584
Bins, s *vs* Concentration, µg L^{-1}	140	13281.2	3.7	< 0.001
Bins, s *vs* Exposure time, h	105	13281.2	1	0.368
Concentration, µg L^{-1} *vs* Exposure time, h	12	379.9	1.1	0.359
Bins, s *vs* Concentration, µg L^{-1} *vs* Exposure time, h	420	13281.2	1	0.467

Legend: bins= 10second time bin during dark and lights on; concentration= concentration of Ag in the water (µg L/1); exposure time= time of exposure in hours; *vs*= analysis of the interaction of the factors.

575

576

577

Table 2. LME statistical analysis for experiment B1. Velocity (cm/s) as dependent variable.

Factors	Numerator df	Denominator df	F	Sig.
Bins, s	35	3814.1	17.1	< 0.001
Treatment	2	110.0	1.1	0.326
Exposure time, d	2	109.9	3.2	0.044
Bins, s*vs*Treatment	70	3814.1	1.1	0.213
Bins, s*vs* Exposure time, d	70	3814.1	2.2	< 0.001
Treatment *vs* Exposure time, d	3	110.0	0.5	0.653
Bins, s*vs* Treatment *vs* Exposure time, d	105	3814.1	1.0	0.399

Legend: bins= 10second time bin during dark and lights on; treatment= type of food (control, AgCl or AgNP); exposure time= time of exposure in days; *vs*= analysis of the interaction of the factors.

578

579

580

581

Table 3. LME statistical analysis for experiment B2. Velocity (cm/s) as dependent variable.

Factors	Numerator df	Denominator df	F	Sig.
Bins	35	7841.3	34.1	< 0.001
Treatment	2	226.1	2.0	0.142
Exposure time, d	3	226.1	5.1	0.002
Bins*vs*Treatment	70	7841.3	1.6	0.001
Bins *vs* Exposure time, d	105	7841.3	1.3	0.012
Treatment *vs* Exposure time, d	6	226.1	0.4	0.888
Bins *vs* Treatment *vs* Exposure time, d	210	7841.3	1.0	0.406

Legend: bins= 10second time bin during dark and lights on; treatment= type of food (control, AgCl or AgNP); exposure time= time of exposure in days; *vs*= analysis of the interaction of the factors.

582